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PNEUMOCOCCAL FIMBRIAL PROTEIN A VACCINES

Abstract:

Abstract of WO 9310238

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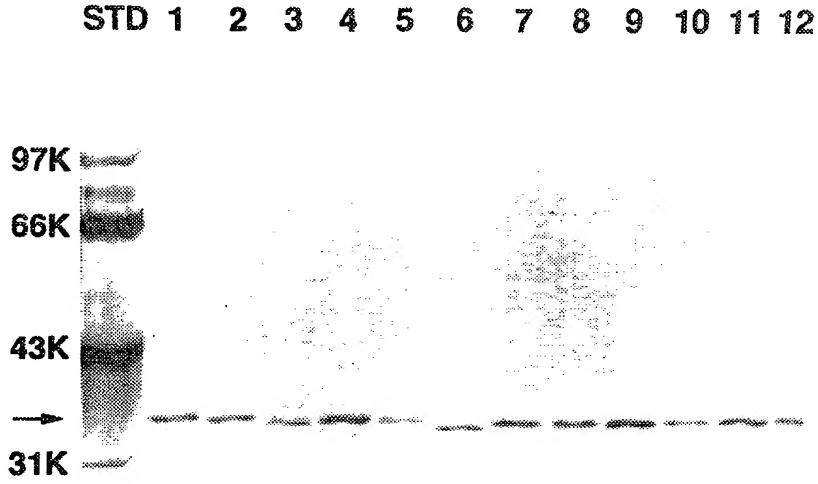

(57) Abstract

The present invention relates, in general, to pneumococcal fimbrial protein A. In particluar, the present invention relates to a DNA segment encoding a pneumococcal fimbrial protein A gene; polypeptides encoded by said DNA segment; recombinant DNA molecules containing the DNA segment; cells containing the recombinant DNA molecule; a method of producing a pneumococcal fimbrial protein A polypeptide; antibodies specific to pneumococcal fimbrial protein A; a method of measuring the amount of pneumococcal fimbrial protein A in a sample, and, vaccines containing pneumococcal fimbrial protein A or a polypeptide derived therefrom.

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PNEUMOCOCCAL FIMBRIAL PROTEIN A VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a Continuation-in-Part of Serial No. 07/791,377 filed September 17, 1991. The entire contents of the above application is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates, in general, to pneumococcal fimbrial protein A (PfpA). In particular, the present invention relates to a DNA segment encoding a pneumococcal fimbrial protein A gene (pfpA); polypeptides encoded by the DNA segment; recombinant DNA molecules containing the DNA segment; cells containing the recombinant DNA molecule; a method of producing a pneumococcal fimbrial protein A polypeptide; antibodies specific to pneumococcal fimbrial protein A; and a method of measuring the amount of pneumococcal fimbrial protein A in a sample.

Background Information

Disease caused by *Streptococcus pneumoniae* (pneumococcus) is an important cause of morbidity and mortality in the United States and developing countries (Sorensen, J. et al. (1986) Scand. J. Infect. Dis. 18:329-335; Wall, R. A. et al. (1986) Bull. WHO 64-4:553-558; Walsh, J. A., and K. S. Warren (1979) N. Eng. J. Med. 301:967-974; Williams, W. W. et al. (1988) Ann. Intern. Med. 108:616-625; Yolken, R. H. et al. (1984) J. Clin. Microbiol. 20:802-805). Pneumococcal disease is very prevalent among the very young, the elderly, and immunocompromised

persons. Despite its prevalence, diagnosis of the disease continues to be a problem.

Several tests have been developed to detect pneumococcus antigens and/or antibodies as a means of diagnosing pneumococcus infections (Coonrod, J. D., and M. W. Rytel (1973) J. Lab Clin. Med. 81:778-786; Holmberg, H. et al. (1985) J. Clin. Microbiol. 22:111-115; Ingram, D. L. et al. (1983) J. Clin. Microbiol. 18:1119-1121; Jalonen, E. et al. (1989) J. Infect. 19:127-134; Kanclerski, K. et al. (1988) J. Clin. Microbiol. 26-1:96-100; Makela, P. H. (1982) Scand.J. Infect. Dis. Suppl. 36:111-113; Perlino, C. A. (1984) J. Infect. Dis. 150:139-144; Sippel, J. E. et al. (1984) J. Clin. Microbiol. 20:884-886; Whitby, M. et al. (1985) J. Clin. Pathol. 38:341-344; Yolken, R. H. et al. (1984) J. Clin. Microbiol. 20:802-805). The sensitivity of existing antigen detection tests utilizing body fluids such as serum and urine, remains very low (Ajello, G. W. et al. (1987) J. Clin. Microbiol. 25:1388-1391; Anhalt, J. P., and P. K. W. Yu (1975) J. Clin. Microbiol. 2:510-515; Bartram, C. E. Jr. et al. (1974) J. Lab. Clin. Med. 83:591-598; Congeni, B. L. et al. (1984) Ped. Infect. Dis. 3:417-419; Coonrod, J. D. (1983) Proceedings of the American Journal of Medicine Symposium, July 28, 1983, Am. J. Med. 75:85-92; Coovadia, Y. B. and K. K. Naidu (1985) J. Clin. Pathol. 38:561-564; Dilworth, J. A. (1975) J. Clin. Microbiol. 2:453-455; Doskeland, S. O., and B. P. Berdal (1980) J. Clin. Microbiol. 11:380-384; Martin, S. J. et al. (1987) J. Clin. Microbiol. 25:248-250), except for antigen detection in

cerebrospinal fluids (Henrichsen, J. et al. (1980) J. Clin. Microbiol. 11:589-592; Ingram, D. L. et al. (1983) J. Clin. Microbiol. 18:1119-1121; Lenthe-Eboa, S. et al. (1987) Eur. J. Clin. Microbiol. 6:28-34; Tilton, R. C. et al. (1984) J. Clin. Microbiol. 20:231-234; Yolken, R. H. et al. (1984) J. Clin. Microbiol. 20:802-805). The measurement of antibody response to pneumolysin by enzyme immunoassay (ELISA) appears to be promising for presumptive etiologic diagnosis (Jalonen, E. et al. (1989) J. Infect. 19:127-134; Kalin, M. et al. (1987) J. Clin. Microbiol. 25:226-229; Kanclerski, K. et al. (1988) J. Clin. Microbiol. 26-1:96-100), but the sensitivity and specificity of the test need improvement.

Although a positive blood culture is diagnostic for pneumococcus disease, most patients with bacterial pneumonia do not have bacteremia (Austrian, R. (1974) Prev. Med. 3:443-445; Austrian, R., and I. Gold (1964) Ann. Intern. Med. 60:759-776; Kalin, M. and A. A. Lindberg (1983) Scand. J. Infect. Dis. 15:247-255). The value of sputum cultures has also been questioned because of contamination of the specimens with pharyngeal flora that can include pneumococci (Barrett-Cooner, E. (1971) Ann. Rev. Resp. Dis. 103:845-848). Thus, clinical laboratories are rarely successful in establishing a firm bacterial etiology for those patients with respiratory infections diagnosed presumptively as pneumococcus pneumonia. Researchers have been in constant search for immunodiagnostic markers or tests to aid in the early diagnosis of pneumococcus infections.

SUMMARY OF THE INVENTION

It is a general object of this invention to provide pneumococcal fimbrial protein A (PfpA) (a 37-kilodalton protein).

It is a specific object of this invention to provide a DNA segment which encodes a pneumococcal fimbrial protein A gene (pfpA).

It is a further object of the invention to provide a polypeptide corresponding to a pneumococcal fimbrial protein A gene (pfpA).

It is another object of the invention to provide a recombinant DNA molecule comprising a vector and a DNA segment encoding a pneumococcal fimbrial protein A gene (pfpA).

It is a further object of the invention to provide a cell that contains the above-described recombinant molecule.

It is another object of the invention to provide a method of producing a polypeptide encoding a pneumococcal fimbrial protein A gene (pfpA).

It is a further object of the invention to provide antibodies having binding affinity to a pneumococcal fimbrial protein A gene (pfpA), or a unique portion thereof.

It is a further object of the invention to provide a method of measuring the amount of pneumococcal fimbrial protein A in a sample.

It is an additional object of the invention to provide vaccines capable of providing protection against pneumococcal pneumonia containing a pharmaceutical effective amount of

pneumococcal fimbrial protein A (PfpA) or polypeptides derived therefrom and methods of administrating those vaccines.

Further objects and advantages of the present invention will be clear from the description that follows.

In one embodiment, the present invention relates to a DNA segment coding for a polypeptide comprising an amino acid sequence corresponding to a pneumococcal fimbrial protein A gene.

In another embodiment, the present invention relates to a polypeptide free of proteins with which it is naturally associated and comprising an amino acid sequence corresponding to a pneumococcal fimbrial protein A gene.

In a further embodiment, the present invention relates to a recombinant DNA molecule comprising a vector and a DNA segment that codes for a polypeptide comprising an amino acid sequence corresponding to a pneumococcal fimbrial protein A gene.

In yet another embodiment, the present invention relates to a cell that contains the above-described recombinant DNA molecule.

In a further embodiment, the present invention relates to a method of producing a polypeptide comprising an amino acid sequence corresponding to a pneumococcal fimbrial protein A gene.

In yet another embodiment, the present invention relates to an antibody having binding affinity to a polypeptide

encoding a pneumococcal fimbrial protein A gene, or a unique portion thereof.

In a further embodiment, the present invention relates to a method of measuring the amount of pneumococcal fimbrial protein A in a sample, comprising contacting the sample with the above-described antibodies and measuring the amount of immunocomplexes formed between the antibodies and any pneumococcal fimbrial protein A in the sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Immunoblot of *S. pneumoniae* whole-cell antigen preparations with pneumococcus MAbs. Protein standards (STD) (in kilodaltons) and different serotypes of *S. pneumoniae* are shown. Lanes: 1, serotype 3; 2, serotype 6B; 3, serotype 7F; 4, serotype 8; 5, serotype 9V; 6, serotype 10A; 7, serotype 11A; 8, serotype 12F; 9, serotype 15B; 10, serotype 19A; 11, serotype 19F; 12, serotype 22F. The MAbs revealed an antigen at 37 kDa (arrow) in all serotypes tested.

Figure 2. Immunofluorescence assay staining of *S. pneumoniae* cells with pneumococcal MAbs.

Figure 3. Transmission electron microscopy of *S. pneumoniae* R36A after embedding, cutting, reacting with Mabs, and staining with gold-labeled goat anti-mouse immunoglobulin.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention relates to a DNA segment coding for a polypeptide comprising an amino acid

sequence corresponding to pneumococcal fimbrial protein A, or at least 5 contiguous amino acids thereof. In one preferred embodiment, the DNA segment comprises the sequence shown in SEQ ID NO:1, allelic or species variation thereof, or at least 15 contiguous nucleotides thereof (preferably, at least 20, 30, 40, or 50 contiguous nucleotides thereof). In a further preferred embodiment, the DNA segment encodes the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof, or at least 5 contiguous amino acids thereof (preferably, at least 5, 10, 15, 20, 30 or 50 contiguous amino acids thereof).

In a further embodiment, the present invention relates to a polypeptide free of proteins with which it is naturally associated or a polypeptide bound to a solid support and comprising an amino acid sequence corresponding to pneumococcal fimbrial protein A, or at least 5 contiguous amino acids thereof (preferably, at least 5, 10, 15, 20, 30 or 50 contiguous amino acids thereof). In one preferred embodiment, the polypeptide comprises the amino acid sequence set forth in SEQ ID NO:2, or allelic or species variation thereof equivalent thereto (for example, immunologically or functionally, equivalent thereto), or at least 5 contiguous amino acids thereof (preferably, at least 5, 10, 15, 20, 30 or 50 contiguous amino acids thereof).

In another embodiment, the present invention relates to a recombinant DNA molecule comprising a vector (for example plasmid or viral vector) and a DNA segment (as described above)

coding for a polypeptide corresponding to pneumococcal fimbrial protein A, as described above. In a preferred embodiment, the encoding segment is present in the vector operably linked to a promoter.

In a further embodiment, the present invention relates to a cell containing the above described recombinant DNA molecule. Suitable host cells include procaryotes (such as bacteria, including E. coli) and both lower eucaryotes (for example yeast) and higher eucaryotes (for example, mammalian cells). Introduction of the recombinant molecule into the cell can be effected using methods known in the art.

In another embodiment, the present invention relates to a method of producing a polypeptide having an amino acid sequence corresponding to pneumococcal fimbrial protein A comprising culturing the above-described cell under conditions such that the DNA segment is expressed and the polypeptide thereby produced and isolating the polypeptide.

In yet another embodiment, the present invention relates to an antibody having binding affinity for pneumococcal fimbrial protein A, or a unique portion thereof. In one preferred embodiment, pneumococcal fimbrial protein A comprises the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof, or at least 5 contiguous amino acids thereof (preferably, at least 5, 10, 15, 20, 30 or 50 contiguous amino acids thereof).

Antibodies (monoclonal or polyclonal) can be raised to pneumococcal fimbrial protein A, or unique portions thereof, in

its naturally occurring form and in its recombinant form. Binding fragments of such antibodies are also within the scope of the invention.

Pneumococcal fimbrial protein A may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. Pneumococcal fimbrial protein A or its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See for example, Microbiology, Hoeber Medical Division (Harper and Row, 1969), Landsteiner, Specificity of Serological Reactions (Dover Publications, New York, 1962) and Williams et al., Methods in Immunology and Immunochemistry, Vol. 1 (Academic Press, New York, 1967), for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin is isolated.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts. Description of techniques for preparing such monoclonal antibodies may be found in Stites et al., editors, Basic and Clinical Immunology, (Lange Medical Publications, Los Altos, CA, Fourth edition) and references cited therein, and in particular in Kohler and Milstein in Nature 256:495-497 (1975), which discusses one method of generating monoclonal antibodies.

In another embodiment, the present invention relates to a hybridoma which produces a monoclonal antibody or binding fragment thereof having binding affinity for pneumococcal fimbrial protein A. In one preferred embodiment, the pneumococcal fimbrial protein A has the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof, or at least 5 contiguous amino acids thereof (preferably, at least 5, 10, 15, 20, 30 or 50 contiguous amino acids thereof). In another preferred embodiment, the hybridoma comprises 1E7A3D7C2.

In yet another embodiment, the present invention relates to a diagnostic kit comprising:

i) at least one of the above-described monoclonal antibodies, and

ii) a conjugate comprising a binding partner of said monoclonal antibody and a label.

In a further embodiment, the present invention relates to a diagnostic kit comprising a conjugate comprising:

i) at least one of the above-described monoclonal antibodies, and

ii) a label.

In a further embodiment, the present invention relates to a method of measuring the amount of pneumococcal fimbrial protein A in a sample, comprising contacting the sample with the above-described antibodies and measuring the amount of immunocomplexes formed between the antibodies and any pneumococcal fimbrial protein A in the sample. Methods of

measuring the amount of immunocomplexes formed can be those well known in the art, such as RIA, ELISA, and direct and indirect immunoassays.

In another embodiment, the present invention relates to vaccines comprising the pneumococcal fimbrial protein A and the above-identified polypeptides derived therefrom and to methods of immunizing mammals (e.g., humans) with the vaccines. The presently used commercial vaccine, Pneumovax, is a mixture of 23 capsular polysaccharides from S. pneumoniae. The vaccine is efficacious in adults but not effective in children less than two years of age. Since the polypeptides of the present invention are proteins, they can be used to protect against pneumococcal disease in children and adults. In one preferred embodiment, the pneumococcal fimbrial protein A and the polypeptides derived therefrom described above, may be conjugated to components of existing commercial vaccines. Data indicate that some children less than two years of age produce antibodies to the pneumococcal fimbrial protein A (the 37-Kda protein).

The present invention is described in further detail in the following non-limiting Examples.

EXAMPLES

The following protocols and experimental details are referenced in the Examples that follow:

Bacterial strains. The *S. pneumoniae* strain R36A was kindly provided by D.E. Briles (University of Alabama at Birmingham). Twenty-four serotypes of *S. pneumoniae* were provided by R. Facklam, Centers for Disease Control (CDC), Atlanta, Ga. These serotypes are 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11F, 11A, 12F, 14, 15B, 18C, 19A, 19F, 20, 22F, 23F, and 33F. *Enterococcus avium*, *E. casseliflavus*, and *E. gallinarum* were also provided by R. Facklam. Anaerobic bacteria were obtained from V.R. Dowell, CDC. These included *Bacteroides asaccharolyticus*, *B. fragilis*, *B. intermedius*, *B. thetaiotaomicron*, *Eubacterium lentum*, *Fusobacterium necrophorum*, *F. nucleatum*, *Peptostreptococcus anaerobius*, *P. asaccharolyticus*, *Propionibacterium acnes*, and *Staphylococcus saccharolyticus*. *Branhamella catarrhalis* and *Bordetella parapertussis* were obtained from R. Weaver, CDC. *Mycobacterium tuberculosis* was provided by R.C. Good, CDC. R. Barnes, CDC, provided *Chlamydia pneumoniae*. The following remaining bacteria were from the stock collection of the Immunology Laboratory, CDC: *Bordetella pertussis*, *Enterobacter aerogenes*, *E. agglomerans*, *E. cloacae*, *E. gergoviae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae* (types a-f), *Legionella micdadei*, *L. pneumophila*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *S. equisimilis*, *S. pyogenes*, and group G streptococci.

Production of Mabs. Female BALB/c mice were immunized with whole cell suspensions of *S. pneumoniae* R36A, a rough derivative of the capsular type 2 strain D39 (Avery, O. T. et al. (1944) J. Exp. Med. 79:137-157). The mice were immunized by intravenous injection three times and intraperitoneal injection one time. The maximum number of cells injected at any time was 10^8. Fusion was done on day 25 by using standard procedures (Clafin, L., and K. Williams (1978) Curr. Top. Microbiol. Immunol. 81:107-109). Spleen cells of 4 mice were fused with Sp2/0-Ag14 myeloma cells (Schulman, M. et al. (1978) Nature (London) 276:269-270). Culture fluids of the growing hybridomas were tested for antibodies to *S. pneumoniae* whole cells in an ELISA. A clone designated 1E7A3D7C2 was one of 10 selected for further study. Further references to Mabs in this article refer to hybridoma clone 1E7A3D7C2.

ELISA. Screening of hybridoma culture supernatants was done by ELISA. U-bottom microtitration plates (Costar, Cambridge, Mass.) were sensitized with 50 µl of *S. pneumoniae* whole cell suspension (10^9 CFU/ml) diluted 1:4,000 in 0.1M carbonate buffer, pH 9.6, and kept for 16 h at 4°C. The plates were washed 5 times with 0.9% NaCl containing 0.05% Tween 20 (NaCl-T). Culture supernatants (50 µl) from the fusion plates were added to 50 µl of a solution containing 2% bovine serum albumin (BSA), 10% normal rabbit serum, 0.3% Tween-20, and 0.02% Merthiolate in phosphate buffered saline (PBS), Ph 7.2, (ELISA diluent) (Wells, D. E. et al. (1987) J. Clin. Microbiol.

25:516-521) in the plates and were incubated for 30 min at 37°C. The plates were washed 5 times with NaCl-T. Fifty microliters of goat anti-mouse immunoglobulin horseradish peroxidase conjugate, diluted in ELISA diluent was added to each well. The plates were incubated for 30 min at 37°C. The plates were washed, and 50 μl of 3,3',5,5'-tetramethylbenzidine (0.1 mg/ml in 0.1M sodium acetate, 0.1 M citric acid [Ph 5.7] with 0.005% hydrogen peroxide) was added to each well and incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of 4 M H_2SO_4 and the optical density was read on a Dynatech ELISA Reader (Dynatech Laboratories, Inc., Alexandria, Va.) at 450 nm. An optical density of >0.200 was considered positive.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Tsang et al. (Tsang, V. C. W. et al. (1983) Methods Enzymol. 92:377-391), using an 8% acrylamide resolving gel. Equal volumes of sample buffer (5% SDS-10% 2-mercaptoethanol-20% glycerol in 0.01 M Tris HCL, [Ph 8.0]) and cell suspension containing 2.4 μg protein per μl were mixed, heated at 100°C for 5 min, and a 5-μl portion was applied to 1 of 15 wells. If the final protein content of the portion of sample to be tested was <1.2μg/μl, a volume up to 10 μl of sample was applied to achieve a final concentration of 6 μg of protein per well. Protein concentrations were determined

by the method of Markwell et al. (Markwell, M. A. et al. (1978) Anal. Biochem. 87:206-210), with BSA as the standard.

Proteins separated by SDS-PAGE were either silver stained by the method of Morrissey (Morrissey, J. H. (1981) Anal. Biochem. 117:307-310) or electroblotted onto nitrocellulose (Schleicher & Schnell, Inc., Keene, N.H.). The immunoblot procedure was done according to the method of Tsang et al. (Tsang, V. C. W. et al. (1983) Methods Enzymol. 92:377-391) with slight modifications. The blots were given three 5-min washes with PBS, pH 7.2, containing 0.3% Tween-20 and were gently agitated overnight (16 h) at 25°C. The blots were blocked for 1 h with casein-thimerosal buffer (CTB) (Kenna, J. G. et al. (1985) J. Immunol. Meth. 85:409-419). After three rinses with CTB, the blots were exposed to goat anti-mouse immunoglobulin horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) for 2 h at 25°C. Conjugate dilutions (1:2,000) were made in CTB. The blots were again rinsed three times with CTB and exposed to 3-3'diaminobenzadine-4-hydrochloride in PBS, pH 7.2 (0.5mg/ml), with 0.003% H_2O_2 for 5 min at 25°C. Reactivity was expressed as a visible colored band on the nitrocellulose paper. Low-molecular-mass protein standards (Bio-Rad) were used in PAGE and immunoblotting. Rabbit antisera to the protein standards were used to develop the standards (Carlone, G. M. (1986) Anal. Biochem. 155:89-91). Molecular masses were calculated by the method of Neville and Glossman (Neville, D. M., and H. Glossman

(1974) Methods Enzymol. 32:92-102) using appropriate molecular mass standards.

IFA. A bacterial suspension containing approximately 400-500 CFU per field (10 μl) was allowed to dry at room temperature on each well of acetone-resistant, 12-well (5 mm diameter), glass slides (25 x 75 mm) (Cel-Line Associates, Newfield, N.J.). The slides were then immersed in acetone for 10 min and air dried at room temperature. Mabs were added to the slides, which were incubated for 30 min at 37°C. After incubation, the slides were gently rinsed with PBS and soaked twice at 5-min intervals, blotted on filter paper, and air dried at room temperature. Fluorescein-labeled rabbit anti-mouse immunoglobulin (courtesy of W. F. Bibb, CDC) was then added, and the slides were incubated for 30 min at 37°C. They were then washed twice with PBS and gently blotted on filter paper. Slides were covered with carbonate-buffered mounting fluid, Ph 9.0, and cover slips and were then read with a Leitz Dialux 20 fluorescence microscope equipped with a HBO-100 mercury incident light source, an I cube filter system, a 40x dry objective lens, and 6.3x binoculars (E. Leitz, Inc., Rockleigh, N.J.).

Immunoelectron microscopy. Pneumococcal cells were washed two times with PBS and fixed in a mixture of 1% paraformaldehyde-0.1% glutaraldehyde (freshly made) for 20 min at 4°C. The cells were dehydrated in a graded alcohol series

and then in a 1:1 mixture of absolute ethanol and Lowicryl K4M (Ladd Research Industries, Inc.,Burlington, Vt.) for 1 h at 4°C. The cells were pelleted and suspended in a 1:2 mixture of absolute ethanol and Lowicryl K4M for 1 h at 4°C. They were again pelleted and suspended in Lowicryl K4M (undiluted) for 16 h at 4°C.

The cells were transferred to fresh Lowicryl K4M two times during the next 24-hour period. The Lowicryl K4M-treated cells were imbedded in gelatin capsules, which were placed inside a box lined with aluminum foil. The capsules were hardened by holding them,in the box, 35 cm from a short-wave UV light source for 72 h at -20°C. The box was brought to room temperature, and the capsules were allowed to continue hardening for up to 14 days.

Samples of the capsule were cut into 100-μm thin sections and picked up on nickel grids. Grids containing the sample were placed on a droplet of ovalbumin solution in PBS containing sodium azide (E. Y. Laboratories, Inc., San Mateo, Calif.) for 5 min. The grids (wet) were transferred to a solution of primary Mabs diluted in a solution of BSA reagent (1% BSA in PBS containing 0.1% Triton X-100, Tween 20, and sodium azide) (E. Y. Laboratories) and incubated for 1 h at room temperature or 18 to 48 h at 4°C in a moist chamber. For antibody binding controls, other grids were wetted with Mabs against *Legionella pneumophila*. The grids were rinsed two times with PBS and incubated on droplets of goat anti-mouse IgG-labeled colloidal gold particles (20 μm)(E. Y.

Laboratories) for 1 h at room temperature. The grids were rinsed two times and poststained with osmium tetroxide, uranyl acetate, and lead citrate. The grids were examined with a Philips 410 transmission electron microscope.

CBA/CaHN/J Mice. X-linked immune deficiency (xid) of CBA/N mice as prepared by Wicker, L. S. and I. Seher, Curr. Top. Microbiol. Immunol. 124:86-101 were used to study the protection afforded by the 37 kDa protein.

EXAMPLE 1

Monoclonal Antibodies

Hybridoma clone 1E7A3D7C2 produced MAbs that reacted with a 37-kilodalton (kDa) protein antigen (pneumococcal fimbrial protein A) found in *S. pneumoniae*. The MAbs reacted with an antigen fractionated in SDS-PAGE, yielding a single immunoblot band. This indicates that the MAb reacted with epitopes found only on the 37-kDa antigen (pneumococcal fimbrial protein A). The MAbs produced by the immunization of mice with pneumococcal cells reacted with all pneumococcal strains tested (24 serotypes) to yield a sensitivity of 100%. For specificity, 55 different nonpneumococcal strains of bacteria that can also cause respiratory infections (Donowitz, G. R., and G. L. Mandell (1985) In: Principles and practices in infectious diseases, 2nd ed. (G.L. Mandell, R.G. Douglas, and J.E. Bennett, ed.) John Wiley & Sons, Inc., New York, pp.394-404) were tested for antigens reacting with the MAbs. The latter

strains represented 19 genera and 36 species of bacteria. None of the strains tested reacted with the pneumococcal MAbs, thus yielding a specificity of 100%

Of 44 patients known to have pneumococcus disease, 34 (77%) had antibodies that reacted with the 37-kDa antigen (pneumococcal fimbrial protein A) by Western immunoblot (Fig 1).

The MAbs reacted with whole pneumococcal cells to yield a positive test result in both the ELISA and IFA. Figure 2 shows the bright immunofluorescence of whole pneumococcus cells stained by the MAbs and fluorescein-labeled anti-mouse immunoglobulin in the IFA. Results from both the ELISA and the IFA indicate that the antigen has exposed epitopes on the surface of the cell or that the immunoglobulin and other immunologic reagents are able to penetrate the pneumococcal cell walls.

Several strains of group A streptococci were tested for immunofluorescence after reacting with the pneumococcus MAbs. None of the heterologous bacterial cells fluoresced in this test, indicating that the IFA reaction was specific for pneumococcus cells.

To further determine the location on the cell of the 37-kDa antigen (pneumococcal fimbrial protein A) epitopes reacting with the MAbs, immunolabeling experiments were performed. Figure 3 shows that the cells were typical of gram-positive cocci in the process of division. The figure also shows the reaction of MAbs and colloidal gold-labeled

anti-mouse immunoglobulin G with thin sections of whole pneumococcal cells. A large portion of the antigen appears to be intracellular since there is no coating or layering of the labeled MAbs around the cell. The large patch of colloidal gold staining indicates that the MAbs bound antigen located inside the cell wall. There was no colloidal gold binding to control pneumococci that were exposed to the MAbs against *L. pneumophila*.

EXAMPLE 2

Cloning of the Pneumococcal Fimbrial Protein A Gene

Streptococcus pneumoniae DNA digested with restriction enzyme Sau3A1 was ligated to BamHI digested pUC13 and transformed into *E. coli* TB1. Recombinant clones were identified by colony immunoblot using the 37-kDa monoclonal antibody. The plasmid pSTR3-1 is an example of the pneumococcal fimbrial protein A gene cloned into pUC13.

EXAMPLE 3

Preparation of Purified 37 kDa Protein Antigen

Two methods for preparing the 37 kDa protein are used. (1) Streptococcus pneumoniae is conventionally cultured and the cells harvested. Purified 37 kDa protein antigen (pneumococcal fimbrial protein A) is isolated from the Streptococcus pneumoniae cell mass by extraction with a non-ionic detergent and further purified by ammonium sulfate fractionation and isoelectric focusing. (2) E. coli TB1 strains containing

plasmid pSTR3-1 is cultured conventionally and the cells harvested. For improved yields, E. coli strains, transformed with an expression vector that carries a strong, regulated prokaryatic promoter and which contains the gene coding for the 37 kDa protein, is used. Suitable expression vectors are those that contain a bacteriophage λP_L promoter (e.g., pKK1773-3), a hybrid trp-lac promoter (e.g., pET-3a) or a bacteriophage T7 promoter. The 37 kDa protein (PfpA) is then extracted from the separated cell mass.

PROTECTION EXPERIMENTS WITH 37 kDa PROTEIN

Experiment No. 1

Twenty CBA/CaHN/J mice carrying the *xid* (x-linked immunodeficiency) mutation were used in this protection study. They were tested for protection against challenge with a virulent type 3 *Streptococcus pneumoniae* strain, WU2. Mice were anesthetized with Ketamine/Rompun and bled infraorbitally to obtain pre-immunization sera. 37 kDa protein (pneumococcal fimbrial protein A) was emulsified in complete Freund's adjuvant (CFA) to a protein concentration of 54 μg per ml. Ten mice were injected subcutaneously into 2 axillary and 2 inguinal sites at 0.1 ml per site, delivering approximately 22 μg protein/mouse. Ten control mice were treated identically with CFA and buffer substituting for protein. Fourteen days later, the ten test mice were injected intraperitoneally (IP) with 100 μg of the 37 kDa protein; controls were injected IP with buffer. Eight days following the IP immunizations, all

mice were bled infraorbitally to obtain post-immunization sera, and challenged intravenously (IV) with 60 CFU of a log phase culture of *S. pneumoniae* strain WU2, a virulent capsular type 3 strain. Mice were observed for 21 days, and deaths were recorded.

Sera were collected prior to immunizations to establish baseline exposures,and also following the full immunization protocol (but before challenge) in order to correlate circulating antibody to the 37 kDa protein with protection.

Days post challenge:
1--no deaths
2--3 control mice dead
3--2 control mice dead
4--2 control mice dead, one sick
5--1 control mouse dead
6-21 no deaths

Immunized with 37 kDa protein: 10/10 survived

Controls with no protein: 2/10 survived (8/10 died)

Difference statistically significant:
(p=0.0008) Rank sum test

Experiment No. 2

Twenty CBA/CaHN/J mice carrying the *xid* mutation were injected according to the following protocol:

1. All mice were bled prior to immunization to establish baseline immunity. Ten test mice were immunized subcutaneously in four sites with a total of 21 μg of 37 kDa protein antigen (pneumococcal fimbrial protein A) emulsified in Complete Freund's adjuvant (CFA). Ten control mice were immunized identically with CFA and buffer substituting for the antigen.

2. Fourteen days later, the mice were boosted intraperitoneally (I.P.) with 100 μg of the 37 kDa protein

antigen (test mice) or with buffer (controls). No adjuvant was used with this booster immunization.

3. Eight days later, all mice were bled via the infraorbital sinus and the sera were collected and pooled into the two groups (immunized and controls). At the same time, blood was collected from individual mice to assay for antibody responses.

4. One day later, two additional mice were injected I.O. with 0.1 ml of pooled immune sera to attempt to passively transfer immunity. Three additional mice were injected I.P. with 0.1 ml of pooled control mouse sera. (Only five mice were injected at this step because of the small amount of sera obtained from the immunized mice.)

5. One hour after the I.P. injections, these five mice were challenged intravenously (I.V.) with 140 colony-forming units (CFU) of a mid-log phase pneumococcal type 3 strain, WU2.

6. At the same time, the eighteen (8 test and 10 control)* mice were challenged I.V.with the same culture of WU2.

7. Deaths were tallied daily.

RESULTS:	No. Dead/No. Challenged
Immunized with the 37 kDa protein:	0/8*
Control mice:	10/10
Passive Protection:	
Mice receiving immune sera:	0/2
Mice receiving control sera:	3/3

*Two of ten test mice died of other causes prior to challenged with WU2.

Mice immunized with the 37 kDa protein were protected from fatal challenge with strain WU2, and this immunity could be passively transferred with sera from immunized mice.

Experiment No. 3

An enzyme-linked immunosorbent assay (ELISA) was developed using purified *S. pneumoniae* 37 kDa protein antigen as a capture for human antibodies. Paired sera were tested from children, less than 24 months of age, known to have pneumococcal pneumonia. Disease confirmation was determined by blood culture or antigen in the urine. It was found that 35% (9/26) had antibody titers greater than sera from non-ill children of the same age group, p=0.06. This illustrates that some of the children responded to the 37 kDa protein antigen after natural infection.

PREPARATION OF THE 37 kDa PROTEIN OR POLYPEPTIDE CONJUGATE

Conjugates can be prepared by use of a carrier protein bound to the 37 kDa protein or polypeptides derived from 37 the kDa protein via a linker, to elicit a T cell dependent response. Such carrier proteins could be any immunogenic protein, for example, keyhole limpet hemocyanin, bovine serum albumin, tetanous toxoid, diphtheria toxoid, and bacterial outer membrane proteins. Examples of bacterial outer membrane proteins, useful as conjugates, include outer membrane proteins of *Neisseria meningitidis* and *Haemophilus influenzae*.

Neisseria meningitidis can be an organism selected from *Neisseria meningitidis*, group A, B, or C.

In addition, the 37 kDa protein or polypeptides thereof can be used in a conjugate where the 37 kDa protein or polypeptides thereof are the T-cell dependent immunogenic carrier for polysaccharide antigens that are B-cell stimulators. This is based on the theory that polysaccharide antigens are B-cell stimulators and that protective immunity is usually generated by a combination of B-cell and T-cell stimulation. Protein antigens exhibit T-cell dependent properties; i.e., booster and carrier priming. T-cell dependent stimulation is important because children less than two years of age do not respond to T-cell independent antigens. The attachment or conjugation of antigens can be accomplished by conventional processes, such as those described in U.S. Patent No. 4,808,700, involving the addition of chemicals that enable the formation of covalent chemical bonds between the carrier immunogen and the immunogen.

In use, the 37 kDa protein antigen of this invention can be administered to mammals; e.g., human, in a variety of ways. Exemplary methods include parenteral (subcutaneous) administration given with a nontoxic adjuvant, such as an alum precipitate or peroral administration given after reduction or ablation of gastric activity; or in a pharmaceutical form that protects the antigen against inactivation by gastric juice (e.g., a protective capsule or microsphere).

The dose and dosage regimen will depend mainly upon whether the antigen is being administered for therapeutic or prophylactic purposes, the patient, and the patient's history. The total pharmaceutically effective amount of antigen administered per dose will typically be in the range of about 2μg to 50μg per patient.

For parental administration, the antigen will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles include water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Non aqueous vehicles, such as fixed oils and ethyl oleate, may also be used. Liposomes may be used as vehicles. The vehicle may contain minor amounts of additives, such as substances which enhance isotonicity and chemical stability; e.g., buffers and preservatives.

* * * * * * * * * * * * * * * *

All publications and patents mentioned herein above are hereby incorporated in their entirety by reference. Additionally, Russell et al. (Oct. 1990), J. of Clin. Microbiol. 28:2191-2195 is hereby incorporated in its entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Russell, Harold

(ii) TITLE OF INVENTION: PNEUMOCOCCAL FIMBRIAL PROTEIN A

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: BIRCH, STEWART, KOLASCH & BIRCH

(B) STREET: P.O. BOX 747

(C) CITY: FALLS CHURCH

(D) STATE: VA

(E) COUNTRY: USA

(F) ZIP: 22040-0747

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: GERALD M. MURPHY, JR.

(B) REGISTRATION NUMBER: 28,977

(C) REFERENCE/DOCKET NUMBER: 1173-379P

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (703) 241-1300

(B) TELEFAX: (703) 241-0369
(C) TELEX: 248345

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1175 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 243..1172

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 10                  15

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 20                  25

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120                 125

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130                 135

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His Ala Trp Leu Asn Leu Glu Asn Gly Ile
140                 145

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230                 235

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260                 265

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Lys
310
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 310 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Ser Asp Lys Leu Lys Val Val Thr Thr Asn
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Ser Ile Leu Ala Asp Ile Thr Lys Asn Ile
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Met Ile Val Thr Ser Glu Gly Cys Phe Lys
205 210

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Asp Ser Ile Ala Lys Glu Gly Glu Glu Gly
285 290

Asp Ser Tyr Tyr Ser Met Met Lys Trp Asn
295 300

Leu Glu Lys Ile Ala Glu Gly Leu Asn Lys
305 310

WHAT IS CLAIMED IS:

1. A vaccine capable of providing protection against pneumococcal pneumonia comprising a pharmaceutically effective amount of pneumococcal fimbrial protein A (PfpA) or a polypeptide derived therefrom in pharmaceutically acceptable excipients.

2. A vaccine according to claim 1, wherein the pneumococcal fimbrial protein A or a polypeptide derived from the pneumococcal fimbrial protein A is part of a conjugate with a carrier protein.

3. A vaccine according to claim 2, wherein the carrier protein is selected from the group consisting of keyhole limpet of hemocyanin, bovine serum albumin, a toxoid and a bacterial outer membrane protein.

4. A vaccine according to claim 3, wherein the bacterial outer membrane is selected from the group consisting of outer membrane proteins of *Neisseria meningitidis* and *Haemophilus influenzae*.

5. A vaccine according to claim 1, additionally containing pharmaceutically acceptable adjuvants.

6. A vaccine according to claim 1, wherein the pneumococcal fimbrial protein A or a polypeptide derived therefrom is a carrier conjugated to a polysaccharide antigen.

7. A vaccine according to claim 3, wherin the toxoid is a member selected from the group consisting of tetanus toxoid and diphtheria toxoid.

8. A vaccine according to claim 1, which is capable of eliciting a combination of B-cell and T-cell stimulation.

9. A method of immunizing a mammal against pneumococcal pneumonia by administrating to said mammal a therapeutically effective amount of the vaccine of claim 1.

10. A method of immunizing a mammal against pneumococcal pneumonia by administrating to said mammal a therapeutically effective amount of the vaccine of claim 2.

11. A method of immunizing according to claim 9, wherein the mammal is a human.

12. A method of immunizing according to claim 10, wherein the mammal is a human.

13. A method of immunizing a mammal against pneumococcal pneumonia by administrating to said mammal a therapeutically effective amount of the vaccine of claim 6.

14. A method of immunizing according to claim 13, wherein the mammal is a human.

15. A method of immunizing a mammal against pneumococcal pneumonia by administrating to said mammal a therapeutically effective amount of the vacicne of claim 7.

16. A method of immunizing according to claim 15, wherein the mammal is human.

17. A method of immunizing a mammal against pneumococcal pneumonia by administrating to said mammal a therapeutically effective amount of the vaccine of claim 8.

18. A method of immunizing according to claim 17, wherein the mammal is human.

19. A DNA segment coding for a polypeptide comprising an amino acid sequence corresponding to pneumococcal fimbrial protein A, or at least 5 contiguous amino acids thereof.

20. The DNA segment according to claim 1, wherein said DNA segment comprises the sequence shown in SEQ ID NO:1, allelic or species variation thereof, or at least 15 contiguous nucleotides thereof.

21. The DNA segment according to claim 2, wherein said DNA segment has the sequence shown in SEQ ID NO:1, allelic or species variation thereof.

22. The DNA segment according to claim 3, wherein said DNA segment has the sequence shown in SEQ ID NO:1.

23. The DNA segment according to claim 1, wherein said DNA segment encodes the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof, or at least 5 contiguous amino acids thereof.

24. The DNA segment according to claim 5, wherein said DNA segment encodes the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof.

25. The DNA segment according to claim 6, wherein said DNA segment encodes the amino acid sequence set forth in SEQ ID NO:2.

26. A polypeptide free of proteins with which it is naturally associated and comprising an amino acid sequence corresponding to pneumococcal fimbrial protein A, or at least 5 contiguous amino acids thereof.

27. The polypeptide according to claim 8, wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof, or at least 5 contiguous amino acids thereof.

28. The polypeptide according to claim 9, wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof.

29. The polypeptide according to claim 10, wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO:2.

30. A polypeptide bound to a solid support and comprising an amino acid sequence corresponding to pneumococcal fimbrial protein A.

31. The polypeptide according to claim 12, wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof, or at least 5 contiguous amino acids thereof.

32. The polypeptide according to claim 13, wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof.

33. The polypeptide according to claim 14, wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO:2.

34. A recombinant DNA molecule comprising a vector and the DNA segment according to claim 1.

35. The molecule according to claim 16, wherein said molecule comprises pSTR3-1

36. A cell that contains the recombinant DNA molecule according to claim 16.

37. A method of producing a polypeptide having an amino acid sequence corresponding to pneumococcal fimbrial protein A comprising culturing the cell according to claim 18 under conditions such that said DNA segment is expressed and said polypeptide thereby produced and isolating said polypeptide.

38. An antibody or binding fragment thereof having binding affinity to pneumococcal fimbrial protein A.

39. The antibody according to claim 20, wherein pneumococcal fimbrial protein A has the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof, or at least 5 contiguous amino acids thereof.

40. The antibody according to claim 21, wherein pneumococcal fimbrial protein A has the amino acid sequence set forth in SEQ ID NO:2 or allelic or species variation thereof.

41. The antibody according to claim 22, wherein pneumococcal fimbrial protein A has the amino acid sequence set forth in SEQ ID NO:2.

42. A hybridoma which produces a monoclonal antibody having binding affinity to pneumococcal fimbrial protein A, or binding fragment thereof.

43. The hybridoma according to claim 24, wherein pneumococcal fimbrial protein A has the amino acid sequence set forth in SEQ ID NO:2, allelic or species variation thereof, or at least 5 contiguous amino acids thereof.

44. The hybridoma according to claim 25, wherein pneumococcal fimbrial protein A has the amino acid sequence set forth in SEQ ID NO:2 or allelic or species variation thereof.

45. The hybridoma according to claim 26, wherein pneumococcal fimbrial protein A has the amino acid sequence set forth in SEQ ID NO:2.

46. The hybridoma according to claim 24, wherein said hybridoma comprises 1E7A3D7C2.

A diagnostic kit comprising:

i) at least one monoclonal antibody according to claim 20, and

ii) a conjugate comprising a binding partner of said monoclonal antibody and a label.

47. A diagnostic kit comprising a conjugate comprising:

i) at least one monoclonal antibody according to claim 20, and

ii) a label.

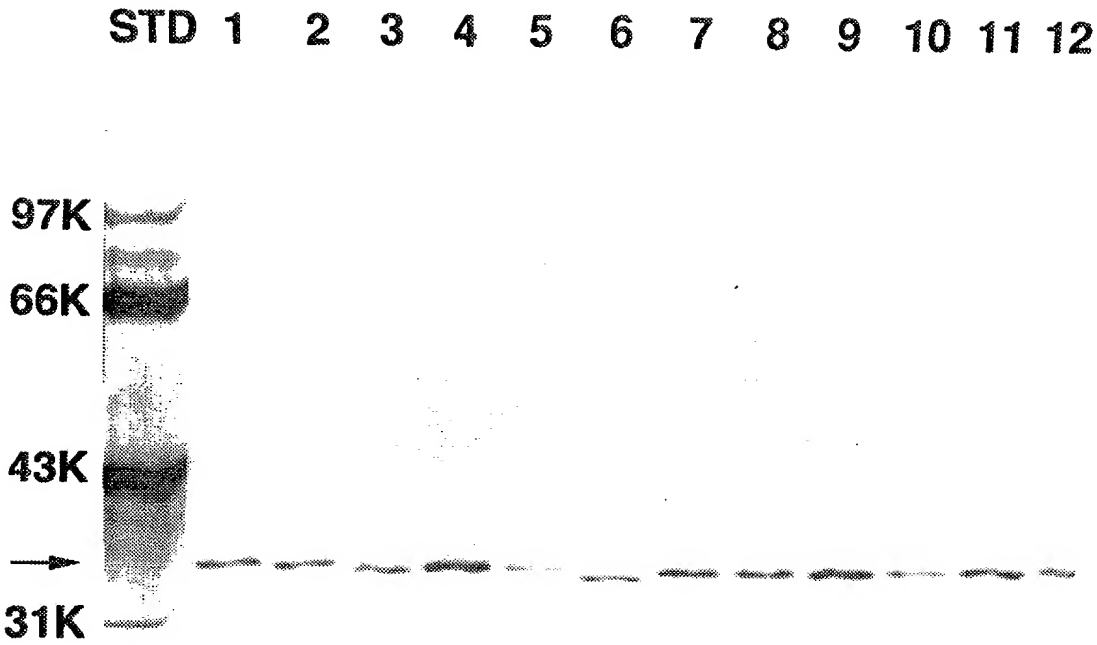

FIG. 1

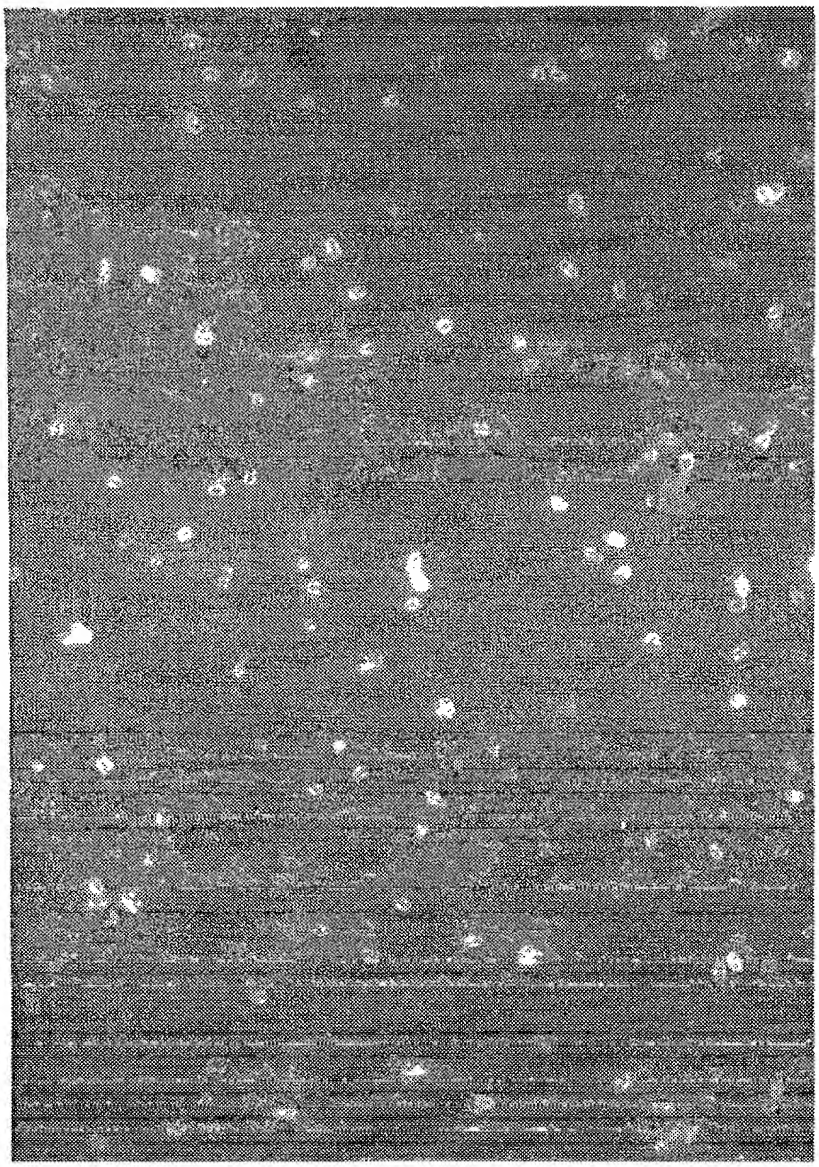

FIG. 2

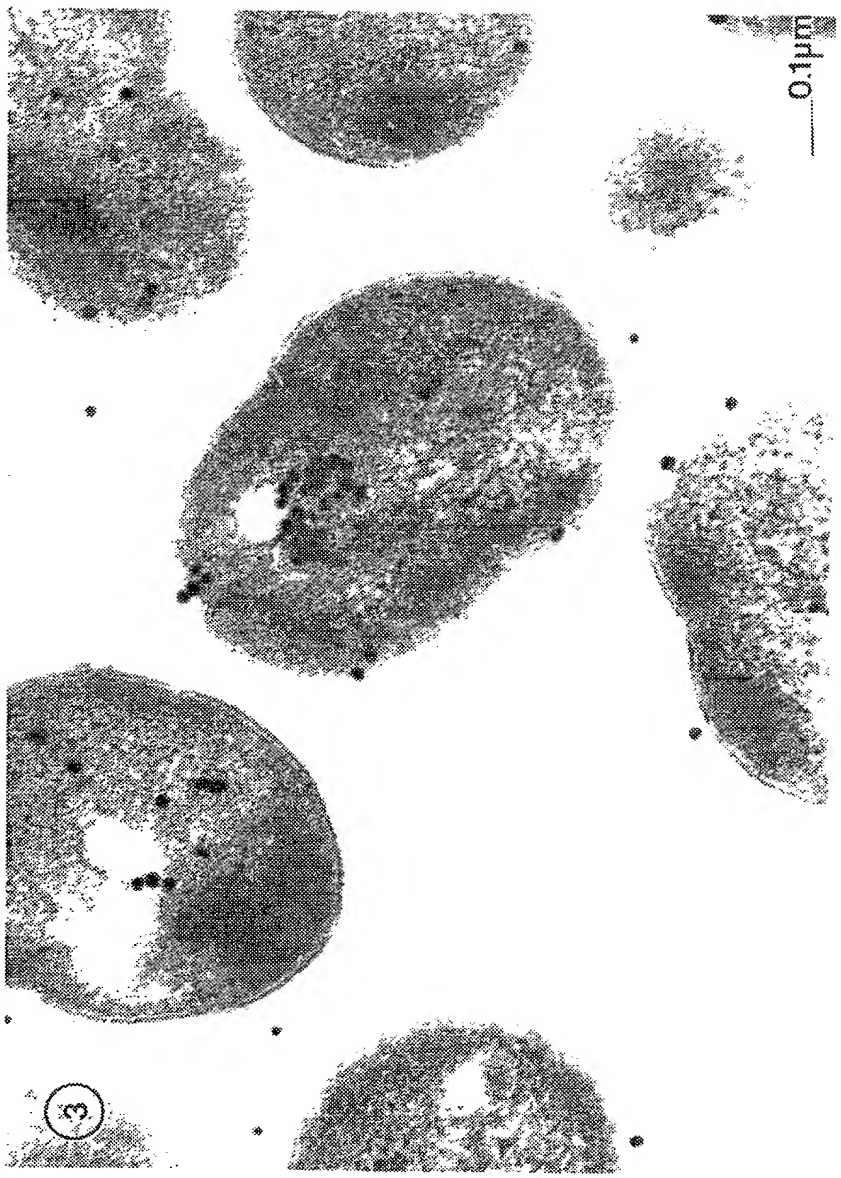

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/09522

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/31; C07K13/00; A61K39/09; A61K39/385
C12P21/02; C12N5/12; C07K15/00; G01N33/577

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; A61K ; C12P G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A,O	ABSTRACTS OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY-1989 1989, WASHINGTON US; ABSTR.NO. D-255 page 125 L.S. MCDANIEL ET AL. 'Molecular Cloning of the Gene Encoding PspA (Pneumococcal Surface Protein A) and Analysis of Protective Immunity Induced by PspA' see abstract & '89th Annual Meeting of the American Society for Microbiology, New Orleans, LA, US, 14-18 May 1989' --- -/--	1-10,13, 15,17, 19-29, 31-34, 36-45

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 FEBRUARY 1993	05. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	THIELE U.H.-C.H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
O,X	ABSTRACTS OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY-1991 1991, WASHINGTON US; ABSTR.NO. D-112 page 97 J. SAMPSON ET AL. 'Molecular Cloning of the Gene Encoding the 37-Kilodalton Protein of Streptococcus pneumoniae' see abstract & '91th Annual Meeting of the American Society for Microbiology, Dallas, Texas, US, 5-9 May 1991' ---	19-25, 34-36, 38-46
O,X	ABSTRACTS OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY-1990 1990, WASHINGTON US; ABSTR.NO. V-19 page 436 H. RUSSELL & J.A. THARPE 'Isolation and Purification of a Species-specific Streptococcus pneumoniae Protein Antigen by Isolelectric Focusing'	26-33, 38-46
Y	see abstract & '90th Annual Meeting of the American Society for Microbiology, Anaheim, CA, US, 13-17 May 1990' ---	1-8
Y	EP,A,0 429 816 (F. HOFFMANN-LA ROCHE AG) 5 June 1991 see claims 1-3,11 ---	1-3,5,7, 8
Y	US,A,4 762 713 (P.W. ANDERSON) 9 August 1988 see claims 1-4 ---	3-4
Y	EP,A,0 206 852 (UNIVERSITE CATHOLIQUE DE LOUVAIN) 30 December 1986 see claim 1 ---	6
O,X	ABSTRACTS OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY-1989 1989, WASHINGTON US; ABSTR.NO. V-22 page 489 H. RUSSELL ET AL. 'Investigation of Streptococcus pneumoniae Components for Immunodiagnostic Markers' see abstract & '89th Annual Meeting of the American Society for Microbiology, New Orleans, LA, US, 14-18 May 1989' --- -/--	26-33, 38-46

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	INFECTION AND IMMUNITY vol. 57, no. 11, November 1989, WASHINGTON US pages 3527 - 3533 J.C. FENNO ET AL. 'Nucleotide Sequence Analysis of a Type 1 Fimbrial Gene of Streptococcus sanguis FW213' see figure 2	19,20,23
X	INFECTION AND IMMUNITY vol. 59, no. 3, March 1991, WASHINGTON US pages 1093 - 1099 N. GENESHKUMAR ET AL. 'Nucleotide Sequence of a Gene Coding for a Saliva-Binding Protein (SsaB) from Streptococcus sanguis 12 and Possible Role of the Protein in Coaggregation with Actinomyces' see page 1094, paragraph 4; figure 2	19,20, 23,26, 27,31
P,O, X	ABSTRACTS OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY-1992 1992, WASHINGTON US; ABSTR.NO. D-191 page 127 H. RUSSELL & J.A. THARPE 'Isolation and Purification of a Species-specific Protein from Streptococcus pneumoniae by Isoelectric Focusing and Continous-Elution Electrophoresis' see abstract & '92th Annual Meeting of the American Society for Microbiology, New Orleans, LA, US, 26-30 May 1992'	1,9, 26-29, 31-33, 38-47
O,X	ABSTRACTS OF THE ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY-1991 1991, WASHINGTON US; ABSTR.NO. V-22 page 434 H. RUSSELL ET AL. 'Assay for antibodies against a species-specific Streptococcus pneumoniae antigen in patients with pneumococcal disease' see abstract & '91th Annual Meeting of the American Society for Microbiology, Dallas, Texas, US, 5-9 May 1991'	26-33, 38-46

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JOURNAL OF CLINICAL MICROBIOLOGY vol. 28, no. 10, October 1990, WASHINGTON D.C., US pages 2191 - 2195 H. RUSSELL ET AL. 'Monoclonal Antibody Recognizing a Species-Specific Protein from Streptococcus pneumoniae' cited in the application see page 2192, right column, paragraph 5 see page 2193, left column, paragraph 3 - right column, paragraph 1 see page 2194, left column, paragraph 3	38-47

INTERNATIONAL SEARCH REPORT

International application No
PCT/US92/09522

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 9-18 are directed to a method of treatment of the human/animal body (PCT-Rule 39.1(iv)) the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9209522
SA 66933

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 16/02/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0429816	05-06-91	AU-A- 6557190	09-05-91
		JP-A- 3173830	29-07-91
US-A-4762713	09-08-88	US-A- 4673574	16-06-87
		US-A- 5097020	17-03-92
		US-A- 4761283	02-08-88
		US-A- 4902506	20-02-90
EP-A-0206852	30-12-86	FR-A- 2581877	21-11-86
		AU-B- 598190	21-06-90
		AU-A- 5741286	18-12-86
		JP-A- 62026236	04-02-87
		US-A- 4789735	06-12-88